Self-Incompatibility System of *Oenothera organensis* for the Detection of Genetic Effects at Low Radiation Doses

by K. Sree Ramulu,* Hannelore Schibilla,† and P. Dijkhuis*

The self-incompatibility system of *Oenothera organensis* is used to analyze the frequency of S locus mutations induced by low doses (2.5–20 rad) of fast neutrons and x-rays. The principles and methods of the screening system for detection of low dose effects have been presented.

The results show that low doses induced significantly higher frequencies of seeds and seedlings than those produced spontaneously. The analyses of part of the progenies derived from the control and treated series suggested that they were due to revertible mutations. A modified *in vitro* method of culturing pollinated cut styles has been developed for rapid screening of compatible pollen tubes.

Introduction

Genetic effects of low doses of radiation are attracting more attention, because of public concern in the increasing use of nuclear energy. In higher organisms, analyses of frequencies of mutation induced by low doses of radiations are difficult, because they require extremely large populations for the detection of a statistically significant increase in the mutation frequency above the spontaneous level. The establishment of the dose-effect relationships and RBE values at low dose ranges requires the development of special test-systems.

Certain test systems in higher plants have already been used for radiation studies at low doses, namely somatic mutations for flower color in Tradescantia (1–4) and the chlorotic spots in *Nicotiana tabacum* (5, 6), and gametic mutations for the waxy character in pollen grains of barley and maize (7–11).

The self-incompatibility system in monofactorial gametophytic species of higher plants, which is based on the rejection of incompatible pollen tubes,

bearing the same S alleles as those present in the pistil, by stylar barrier can be a unique test-system for detecting and analyzing the frequencies of mutation induced at low radiation doses. The S locus has a tripartite structure (12, 13): two activity parts control the reaction in the pollen and in the style respectively, and one specificity part, common to both pollen and style, which determines the specificity. It is uniquely suitable for analysis of different types of mutations:

(1) self-compatibility mutations, namely stylar-part or pollen-part mutation resulting from negative changes (deletions or inactivation) in pollen-or stylar-part of the S locus, and

(2) new self-compatibility alleles resulting from a constructive (positive) modification in the specificity part of the S locus (14). These modifications are, when they lead to a breakdown of the incompatibility relationship, automatically screened by the stylar barrier, and selected out of millions of individual cells for transmission to the next generation. Thus, extremely large number of individuals can be analyzed.

With respect to the mapping of S locus in higher plants, the genetic segregation of S alleles and the involvement of chromosomal aberrations in the manifestation of self-compatibility mutations clear-

* Foundation Institute for Atomic Sciences in Agriculture, Wageningen, The Netherlands.

† Université Catholique de Louvain, Agronomy Faculty, Louvain-la-Neuve, Belgium.

ly suggest a chromosomal location of self-compatibility loci, but little information is available to date on the site of the S gene within the genome of a self-incompatible plant. The attempts made to identify S-bearing chromosome in Lycopersicon and Nicotiana have been recently reviewed by de Nettancourt (14).

The S system of *Oenothera organensis* exhibits rigorous incompatibility, i.e., complete absence of pseudo-fertility (15, 16). Lewis (16–18) demonstrated a close agreement between the pollen tube growth method (compatible pollen tubes in the style) and seed-set method for determining mutation rates at the S-locus of *O. organensis*.

Furthermore, because of its long style and due to pollen tube growth inhibition at or near the stigma, *O. organensis* can also be a suitable material to develop biological screening methods for pollen mutated at the S-locus by *in vitro* culture of pollinated style segments (19, 20).

Research with radiations on the one locus gametophytic system has been so far limited to the study, at high doses, of physiological and genetic effects of acute and chronic irradiations (14, 21).

The present article reports on the principles and methods used for the detection and analysis of genetic effects of low doses (2.5, 5, 10, 20 rad) of fast neutrons and x-rays, the results obtained so far on the frequencies of S mutations as determined from the number of seeds and seedlings, and some attempts made to develop a semi *in vitro* screening method for pollen mutated at the S locus.

Material and Methods

Genotype

To deal with a genetically homogeneous population, of *Oenothera organensis* a self-incompatible diploid clone III-4/55 was established for a single S_3S_4 plant by stem-cutting technique (22). The plant (S_3S_4) was obtained from seeds kindly provided by Prof. A. Hecht.

The clone, III-4/55 has an extremely good capacity for vegetative progagation. The propagation and planting were carried out under controlled conditions (16 hr day, 12,000 lux, 23°C; 8 hr night, 17°C; relative humidity 70–80%). Under these conditions, the plants flower almost throughout the year.

Irradiation

The source of fast neutrons was the BARN-reactor at ITAL, Wageningen, The Netherlands. The reactor was set at 800 W and the irradiation treatments were given at a dose rate of 0.17 rad/min the γ-contamination was approximately 3% on Gy basis. The x-irradiation was applied with a Philips deep-therapy apparatus operating at 160 kVp and 9 mA with an additional 0.25 Cu 1.0 Al 0.4 Sn filter. The dose rate was 0.25 rad/min.

Treatment of Pollen Mother Cells (PMC)

Since S gene mutations induced after the tetrad stage are too late to express themselves in the pollen (18, 23), the irradiation treatments were applied to meiotic cells. The flowering shoots of *Oenothera organensis* have a graded developmental series of buds, the youngest being at the tip and the oldest at the base of the shoot. Under our conditions of growing, about 9 mm flower bud size coincides with prophase and 14 mm bud size with tetrad stage. Buds that were 9–13 mm long at the time of irradiation were marked and the pollen from these flowers was used. For plants in the control series, the same procedure was followed. The marked buds opened into flowers after 19–22 days in the control and irradiated series.

Pollination Procedures and Detection of Irradiation Effects

Totals of 585 and 417 flowering plants were treated with fast neutrons and x-rays, respectively. The control series consisted of 600 plants. Five to fifteen plants of the same age at each dose were irradiated each time (replication), the interval between one replication and the other being two or more weeks. In all, 34 replications in fast neutron series and 23 replications in x-ray series have been carried out.

To transmit modifications in the self-compatibility character, mature pollen from flowers which had been in meiosis at the time of irradiation, was applied to the stigmas of flowers of untreated plants from the same S_3S_4 clone. All the pollinations have been made by the method of Lewis (17): pollen from two anthers was applied on the receptive surface of each stigma in a thin layer.

Attempts were made to score the effects of irradiation on the pollen component of the S-gene because large-scale scoring is possible.

To prevent abscission of flowers and to induce swelling of the ovaries, 1% naphthalene acetamide in lanolin was applied around the calyx of the flowers immediately after pollination (17, 24). Every capsule, whether big or small, which developed after pollination with the pollen from treated or untreated plants has been harvested and checked for seed-set.

The seeds were sown in petri dishes on a Perlite medium (soaked with water) and germinated under controlled conditions as described above. The seedlings at the 2–4 leaf stage were transplanted into soil in small pots. At anthesis the plants were self-pollinated and test-crossed with the mother clone.

Estimation of Pollen Grains on Pollinated Stigmas

To determine the mutation rate it is necessary to know the number of pollen grains that are being tested. To do this, the mean number of pollen grains per pollinated stigma must be estimated. The dilution method described by Lewis (17) for O. organensis was used with some modifications. A mixture of 1:3.7 parts (by volume) of absolute alcohol and chloroform has a density in which the pollen grains remain suspended. Two pollinated stigmas were placed in a flask containing 10 ml of the mixture and two samples of 1 ml were withdrawn by pipet from each flask and placed on a calibrated slide for counting. The pollen from two anthers was applied on the surface of each stigma in a thin layer (approximate monolayer). Estimation of pollen grains was made from samples of about 20 stigmas collected periodically. The pollinations were made by one person. The number of aborted pollen grains was subtracted while estimating the number of tested pollen grains for the treated and control series. The mean number of pollen grains per stigma was 4483 ± 121.

Estimation of Mutation Rates

Several factors can influence the estimation of mutation rates, the important ones being the number of pollen grains deposited on a stigma, time of S gene action and the type of self-incompatibility system of the species.

The number of pollen grains accommodated on a stigma can be influenced by several variables (25) which may be overcome to a certain extent by adopting a standard pollination technique, by growing the plants under controlled conditions and by performing experiments each time (replication) with a few selected plants; the replications well spaced in interval.

The time of S gene mutation during microsporogenesis is another important variable. If a mutation has been induced in a late stage of pollen development, there will be only one mutated pollen grain and hence one pollen tube in the style or one seed in the capsule. The earlier in development the mutation is induced the higher will be the number of

mutated pollen grains, hence the larger number of seeds in the capsule. For comparative analyses of mutation frequencies treatments can be applied to buds in a particular stage, e.g., post pachytene (but before tetrad),being the most sensitive stage: a mutation at this stage results in a single seed per capsule.

The species chosen must have a rigorous incompatibility system, so that interference by pseudofertility during scoring procedures can be avoided.

In the present study all these factors have been considered for analyses of frequencies of mutations. Yet, the number of seeds or seedlings determined per total number of pollen grains tested may be considered as approximate estimates.

Pollen Fertility

Pollen stainability was assessed by tests with Alexander's stain (26). The counts were based on 200 pollen grains per flower and 1–2 random flowers which were in meiosis at the time of irradiation per plants. In all, 40 plants in the control and 30 plants per dose in the treated series were analyzed.

Method of Culturing Pollinated Cut Styles for Scoring Compatible Pollen Tubes

The modified in vitro method is briefly summarized below. It involves incubation at 32°C in dark for 30 min, of detached flowers (without any self-pollen on the stigmas) from plants grown under controlled conditions; pollination of flowers followed by incubation at 32°C in dark for 4 hr; collection of the styles from flowers; cutting with a sharp razor the first 60 mm of style (with stigma) in the culture medium; planting the style segment on the medium, the cut-end (½–1 cm) being in the medium in the Petri dish; incubation of petri dishes at 32°C for about 20 hr; counting of pollen tubes grown from the cut-ends into the medium with the aid of a stereomicroscope.

The culture medium adopted by Brewbaker and Kwack (27) for pollen germination in several plant species was slightly modified and tested for pollen germination and tube growth in the clone III-4/55 of O. organensis (28) and also used in the present study for culturing the pollinated cut-styles. It has the following composition: 10% sucrose, 300 ppm $Ca(NO_3)_2 \cdot 4H_2O$, 200 ppm $MgSO_4 \cdot 7H_2O$, 100 ppm H_3BO_3, 100 ppm KCl, 0.5% agar.

Calculations

The frequency of seeded capsules was calculated per 100 pollinations and the frequency of seeds per

pollination. The statistical analysis is based on the assumption, that the variable (the number of seeded capsules and seeds) follow a Poisson distribution with parameter $N\lambda$, where N is the number of flowers pollinated and λ is the expectation of the variable (capsules or seeds) per pollinated flower.

Results

Pollen Abortion

The treatments with low doses of fast neutrons and x-rays significantly ($p < 0.01$) increased pollen abortion (Fig. 1). Percentage abortion increased with increasing dose, being significantly higher following treatment with 20 rad fast neutrons than after 2.5–10 rad fast neutrons or 2.5–20 rad x-rays.

Frequencies of Capsules and Seeds

The frequencies of seeded capsules and seeds after pollination of unirradiated S_3S_4 plants with the pollen from the irradiated or unirradiated S_3S_4 plants are given in Table 1. In the control the mean frequency of seeds per pollination was 0.027. The low doses of fast neutrons and x-rays induced significantly higher frequencies of seeded capsules, and seeds than those produced spontaneously.

Within the dose range of 2.5–20 rad x-rays, no significant differences in the frequencies of seeded capsules or seeds were observed. In the case of fast neutron treatment (2.5–10 rad), the frequencies of
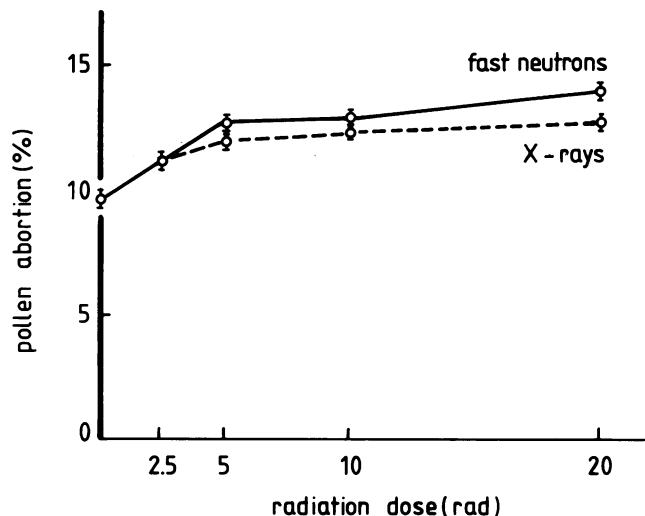

FIGURE 1. Percentage pollen abortion after treatments of meiotic buds with low doses of fast neutrons and x-rays in S_3S_4 of *Oenothera organensis*.

seed (viable and nonviable) decreased with increasing dose ($p = 0.04$), but the number of viable seeds did not differ significantly.

Capsules with Seeds

Table 2 gives the number of capsules containing 0, 1, or more seeds. In all, 10,250 capsules were checked for seed-set. Among these, the single-seeded capsules which occurred in a high frequen-

Table 1. Frequencies of capsules and seeds after crosses between uniradiated S_3S_4 plants, used as female parents and irradiated and control S_3S_4 plants in *Oenothera organensis*.

Irradiation treatments, rad	Flowers pollinated	Capsules With seeds No.	No. per 100 pollinations ± S.E.	With viable seeds No.	No. per 100 pollinations ± S.E.	Seeds No.	Frequency Per pollinations ± S.E.	Per million pollen grains	Viable seeds No.	Frequency Per pollination ± S.E.	Per million pollen grains
Control	5380	54	1.00 ± 0.14	23	0.43 ± 0.09	147	0.027 ± 0.002	6.75	44	0.008 ± 0.001	2.02
Fast neutrons											
2.5	1081	51	4.72 ± 0.66[a]	17	1.57 ± 0.38[a]	60	0.055 ± 0.007[a]	13.97	17	0.016 ± 0.004[a]	3.96
5	1056	36	3.41 ± 0.57[a]	15	1.42 ± 0.37[a]	37	0.035 ± 0.006[c]	8.95	15	0.014 ± 0.004[b]	3.63
10	889	26	2.92 ± 0.57[a]	11	1.24 ± 0.37[a]	33	0.037 ± 0.006[c]	9.50	11	0.012 ± 0.004[c]	3.17
X-Rays											
2.5	455	15	3.30 ± 0.85[a]			24	0.053 ± 0.011[a]	13.26			
5	479	20	4.17 ± 0.96[a]			28	0.058 ± 0.012[a]	14.84			
10	480	17	3.54 ± 0.86[a]			25	0.052 ± 0.010[a]	13.25			
20	430	15	3.49 ± 0.90[a]			26	0.060 ± 0.012[a]	15.38			

[a] $0.01 < p < 0.025$ [b] $0.025 < p < 0.05$ [c] $0.05 < p < 0.10$

Table 2. Frequencies of capsules with varying number of seeds after crosses bet..een unirradiated S_3S_4 plants, used as female parents and irradiated and control S_3S_4 plants in *Oenothera organensis*.

Seeds per capsule	Control	Fast neutrons			X-Rays			
		2.5 rad	5 rad	10 rad	2.5 rad	5 rad	10 rad	20 rad
0	5326	1030	1020	863	440	458	463	415
1	32	43	35	22	10	14	15	12
2	8	7	1	2	2	4	1	1
3–4	9	1	0	2	3	2	0	1
>4	5	0	0	0	0	0	1	1

FIGURE 2. Style, capsules, and seeds in S_3S_4 of *Oenothera organensis*: (a) style with ovary an stigma; (b) capsule formed after a cross-compatible pollination; (c) capsule as in (b), showing seeds after opening; (d) single-seeded capsule development after pollination with the pollen of irradiated plants; (e) capsule as in (d), showing a seed after opening.

cy, were much smaller in size than those produced after a compatible pollination (Fig. 2). From the 180 seeded capsules produced by low radiation doses, 84% had 1 seed, 10% had 2 seeds, and 6% had 3 or more seeds. In the case of control series, of the 54 capsules, a high percentage (60%) also had one seed, 15% had 2 seeds, and 25% had 3 or more seeds.
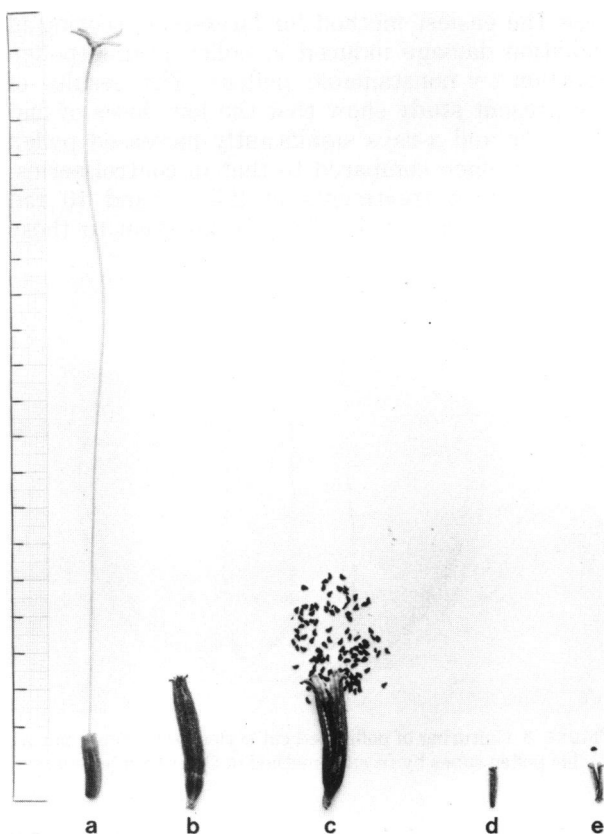
Capsules with Seedlings

Data on germination are not yet available for the x-ray series. In the fast neutron series, 90% of the capsules, which gave rise to seedlings were single-seeded type (Table 3). Among the spontaneously generated capsules that produced seedlings, 70% were of the type with one or two seeds; the other had three or more seeds.

Progeny Testing for S Mutations

Of the 277 seeds, 87 germinated. These eventually gave rise to 61 mature plants, the others died at different stages of the growth and development (Table 4). Xantha seedlings occurred often in the progenies, because the clone III-4/55 (S_3S_4) is heterozygous for Xantha. Some morphological variants were also found which were characterized by having grasslike leaves, leaves with anthocyanin pigmentation, and by poor growth and development.

From the 61 surviving plants, 43 have been so far tested for S mutations, 20 being from the fast neutron series and 23 from the control. All were highly fertile and normal in growth and morphology. The results of self-pollinations and reciprocal crosses with the original mother clone (S_3S_4) showed that all the 43 progeny plants were both self-and cross-incompatible. Thirty-six plants were S heterozygotes, i.e., S_3S_4, and seven S homozygotes. The data of the crosses among the homozygotes indicated that all contained the same S allele, S_3 or S_4, in homozygous condition; it is yet to be determined if all are of S_3S_4 or S_4S_4 constitution.

Table 3. Frequencies of capsules with varying number of viable seeds after crosses between unirradiated S_3S_1 plants, used as female parents and irradiated and control S_3S_1 plants in *Oenothera organensis*.

Seeds per capsule[a]	No. of capsules with viable seeds			
		Fast neutrons		
	Control	2.5 rad	5 rad	10 rad
1	11	16	14	9
2	5	1	1	1
3–4	3	0	0	1
>4	4	0	0	0

[a] Viable and nonviable seeds.

Modified *in vitro* Method of Culturing Pollinated Cut Styles

The basic requirements of the method are to allow the compatible (mutated) pollen tubes to grow out of the basal ends of the cut styles into culture medium and to inhibit all the incompatible pollen tubes near the stigma. The effects of some factors have been investigated, namely temperature (25, 32, 37°C), duration of incubation (1, 2, 4 hr) and the length of cut styles (20, 40, 60 mm). Three or four pollinated cut styles were placed in each petri dish and the number of pollen tubes which grew from the cut ends were scored (Fig. 3). The optimum conditions for the screening method are: an incubation temperature of 32°C, a 4-hr period of post-pollination incubation and 60 mm long cut styles.

In all, 755 flower after self-pollination of S_3S_4 have been scored by a modified *in vitro* method in several replications. As can be seen from the results given in Table 5, the mean number of pollen tubes per pollination was 0.21 ± 0.02. Among the styles that carried pollen tubes, a high percentage (60%) contained a single pollen tube (Fig. 4), the others having 2 or more pollen tubes.

Under the conditions of the screening method, cross-compatible pollinations ($S_3S_4 \times S_{27}S_{28}$) gave an average of about 60 pollen tubes per pollination.

This method can be of use for a rapid scoring of genetic and physiological effects of chemical mutagens and radiations.

Discussion

Effects of Low Radiation Doses on Pollen Abortion

Pollen stainability although not necessarily identical to pollen fertility, is from a practical point of view the easiest method for large-scale scoring of radiation damage induced in pollen grains (pollen abortion $\approx$ nonstainable pollen). The results of the present study show that the low doses of fast neutrons and x-rays significantly increased pollen abortion when compared to that in control series. Fast neutron treatments at 2.5, 5, and 10 rad induced similar levels of pollen abortion to those
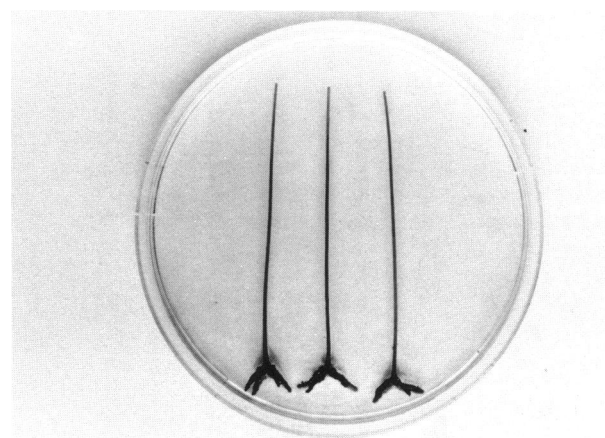
FIGURE 3. Culturing of pollinated cut styles for scoring compatible pollen tubes by *in vitro* method in *Oenothera organensis*.

Table 4. Germination, type and number of plants after crosses between unirradiated S_3S_4 plants, used as female parents and irradiated and control S_3S_4 plants in *Oenothera organensis*.

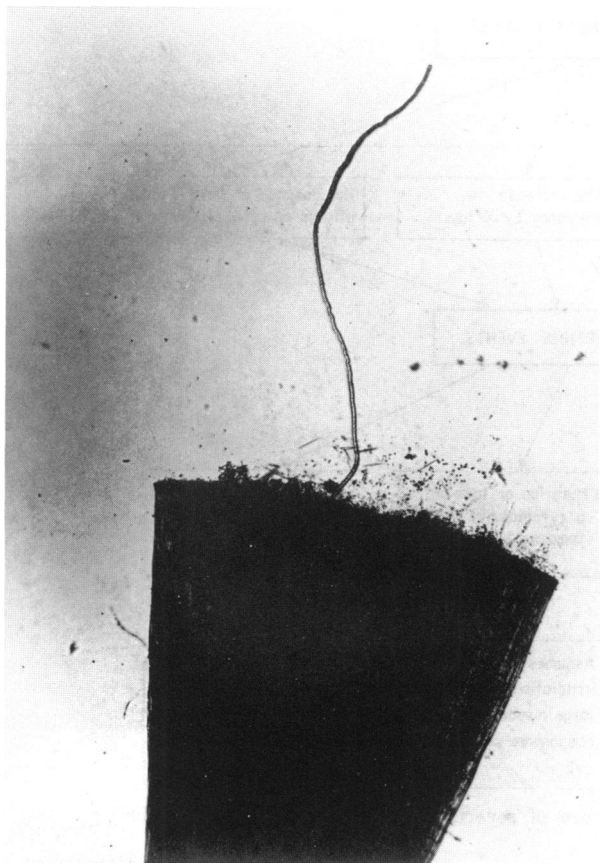| Irradiation treatment, rad	Seeds sown	Seeds germinated	Type and no. of seedlings[a]		
			Normal	*Xantha*	Morphological variants
Control	147	44	39 (11)	3 (3)	2 (1)
Fast neutrons					
2.5	60	17	15 (3)	0	2
5	37	15	11 (1)	2 (2)	2 (2)
10	33	11	9 (1)	2 (2)	0

[a] Number of lethals are given in parentheses.

FIGURE 4. Single pollen tube growing from the basal of the cut end of a style into the culture medium scored by *in vitro* method in *Oenothera organensis*.

induced by 2.5–10 rad x-rays, but were highly effective.

Spontaneous and Induced Mutations at the S locus of *O. organensis*

Because of rigorous incompatibility in *O. organensis*, a close agreement exists between the pollen tube growth method and seed-set method for determining mutations rates at the S locus (16–18). Therefore, the frequencies of S locus mutations can be analyzed at three different levels of viability, as has been done by Lewis (16–18) in different S genotypes of *O. oganensis:* (1) the number of compatible pollen tubes, which gives a maximum measure of the proportion of pollen grains with a changed incompatibility reaction, including those with an inviable sperm nucleus, (2) the number of seeds, which gives an estimate of the number of pollen grains with a mutated S gene and at the same time a sperm nucleus which is able at least to stimulate the initation of a zygote and (3) the

number of seedlings, which gives an estimate of the S mutations which are fully viable.

When compared to the spontaneously produced frequencies of seeds per million pollen grains reported by Lewis (17) for three clones of *O. organensis*, the present clone which differs in origin and S genotype showed a higher frequency of seeds. In regard to the frequencies of seedlings, although the frequencies of capsules with viable seeds were similar, the number of seedlings per million pollen grains differed, being higher than that found by Lewis (16) in a clone which was similar in S genotype, i.e. S_3S_4. Differences in the spontaneous mutation rates between different clones on S genotypes have been previously demonstrated by several studies in various species (16, 17, 24, 29).

Concerning the effects of low radiation doses on the S locus, the results obtained show that both fast neutrons and x-rays produced significantly higher frequencies of seeds and seedlings than those obtained spontaneously. The frequencies doubled after treatment with 2.5 rad. However, the present data, which are incomplete concerning the screening of populations for mutations and determination of the frequency and type of mutations, do not permit a conclusion on dose-effect relationships. The studies by Lewis (16–18) on spontaneous and induced S gene mutations in five different S genotypes of *O. organensis* revealed mutations of two types: permanent self-compatible mutations, namely pollen-part mutation resulting from negative changes (deletions or inactivation) in pollen-part of the S locus; and revertible events. The arguments to demonstrate the genetic nature of revertible events and the four possible explanations given by Lewis (16) for supporting the hypothesis that revertible mutations are summarized in Figure 5 (21). Lewis (16) and Pandey (30, 31) considered that such mutations result from labile premutations which subsequently revert, after a certain number of cell divisions to the original allelomorph.

Further, Lewis (16) demonstrated the novelty of revertible mutations in a clone, which was similar in S genotype (S_3S_4) to that used in the present study. The S_3 allele did not give even a single case of permanent or revertible mutation either spontaneously or after x-irradiation in spite of screening

Table 5. Frequency of pollen tubes after incompatible pollinations.

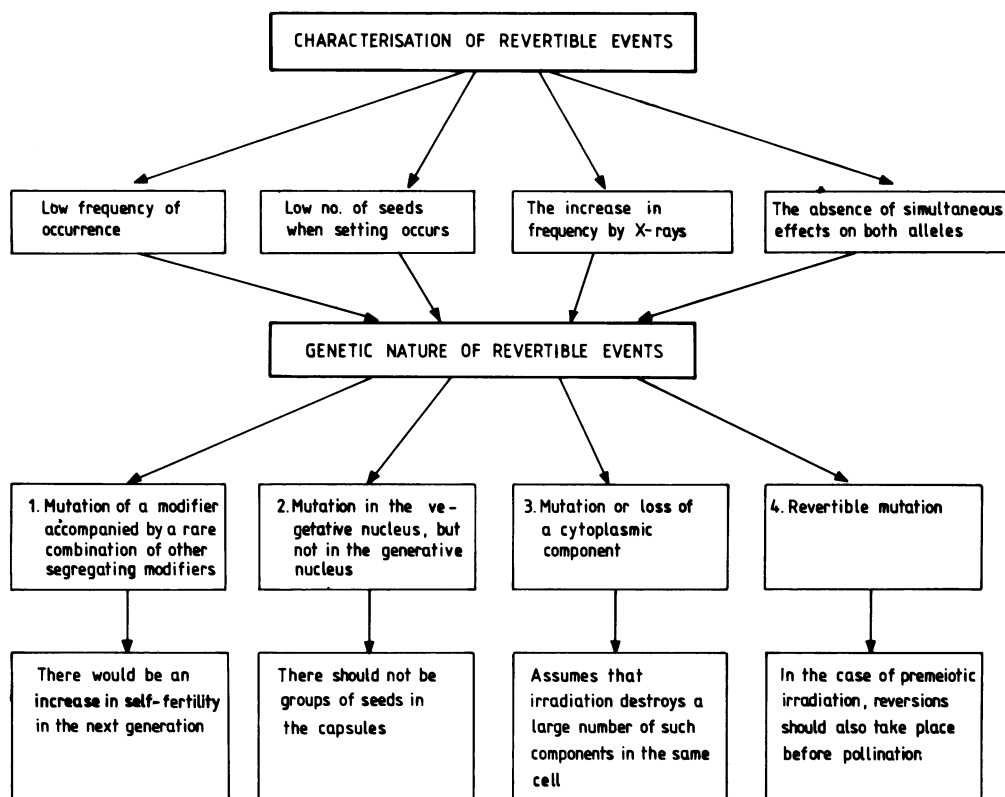| Flowers pollinated	Styles with pollen tubes	Pollen tubes	
		Total no.	No. per pollination (mean ± S.E.)
775	85	162	0.21 ± 0.02

FIGURE 5. Arguments to demonstrate the genetic nature of revertible events and the four possible explanations.

approx. 62 million pollen grains. The S_4 has given spontaneously revertible mutations only, being 0.9 per million pollen grains. But, after x-ray treatments, it gave both revertible and permanent mutations in higher frequencies. The results obtained with S_3S_4 in the present study after self-pollinations and testcrosses of 43 progeny plants showed that they are due to revertible events. Because the data obtained fit Lewis' arguments and explanations (Fig. 5), it can be concluded that these revertible events correspond to revertible mutations. All the seven homozygotes so far tested contained the same allele. However, the present sample is still too small to permit a conclusion on the allele mutability differences. The analysis is being completed.

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